# Acylated β-Caseins: Electrostatic Interactions and Aggregation



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**Abstract**

β-Casein was both acetylated and succinylated, to investigate alterations of selected properties brought about by removal of positive charges and, in the case of succinylated β-casein, addition of negative charges. The results were used to evaluate the role of electrostatic interactions in the aggregation of β-casein. Mobility during alkaline polyacrylamide gel electrophoresis of both derivatives was greater than β-casein, succinylated β-casein having the greatest mobility. Succinylated β-casein and acetylated β-casein, in that order, required a higher concentration of NaCl than did β-casein for elution from DEAE-cellulose at pH 7.0. The calcium ion sensitivity of acetylated β-casein was decreased in comparison to β-casein. Succinylated β-casein was insensitive to calcium ions at pH 7 in 0.1 M $CaCl_2$ at a 0.3% concentration. Sedimentation patterns at pH 6.86, 20 C, revealed that succinylated β-casein did not form a fast-sedimenting peak, usually associated with aggregation. The fast-sedimenting peak of acetylated β-casein had a lower sedimentation coefficient than did β-casein. These alterations of the above properties of β-casein are interpreted to result from an increase in the net negative electrostatic charge of the modified protein in neutral solution. For succinylated β-casein, the net negative electrostatic charge is sufficient to prevent precipitation by calcium ions and to prevent aggregation under conditions that favor aggregation of β-casein.

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Interactions of the caseins responsible for formation of micelles in bovine milk are at this time incompletely understood and characterized. One approach to uncovering some factors that govern protein interactions is to modify a protein chemically and to examine the effect of the modification on some selected property. By blocking amino groups of β-casein with acyl groups, the contribution of electrostatic charge to aggregation of β-casein was investigated. Acetylation of an amino group removes a positive charge from the protein in an acid or neutral solution, whereas succinylation of an amino group adds a negative charge to the protein in a neutral solution in addition to removal of a positive charge. Near neutrality, both acetylated β-casein and succinylated β-casein would be expected to have a larger net negative charge than β-casein. These increases in net negative charge (as much as 11 and 22 for a mol wt of 25,000) should then be reflected in both chromatographic and electrophoretic behavior of the modified protein.

The aggregation of β-casein near neutrality in the presence of calcium ions can be attributed to a decrease in net electrostatic charge resulting from the binding to the protein of positively charged calcium ions (16, 17). In effect, the protein-calcium-ion complex approaches an isoionic point near which precipitation occurs. Whether the increase in net negative charge of the β-casein derivatives could be cancelled by bound calcium ions to an extent that permits aggregation was investigated, to obtain supporting evidence for a possible role for electrostatic charge in the aggregation of β-casein in the presence of calcium ions.

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## Experimental Procedure

*Materials.* β-Casein A was prepared by the urea fractionation method of Aschaffenburg (1). Succinylation of β-casein was performed at room temperature between pH 7 and 8 with succinic anhydride by the method of Hass (4). To a solution of 5 g of $Na_2HPO_4 \cdot 7\ H_2O$ in 250 ml of water was added 2 g of β-casein A. Small portions of a 2-g quantity of succinic anhydride (Eastman)[1] were added with stirring, while the pH was maintained between 7 and 8 by addition of 1 N NaOH. After the pH was stabilized, the solution was filtered, dialyzed against water, and lyophilized. Acetylation of β-casein A was carried out by the method of Fraenkel-Conrat (2). To 120 ml of saturated sodium acetate solution at 0 C was added 2 g of β-casein A. To the vigorously stirred suspension was then added, in 0.2-ml portions, 2.4 ml of acetic anhydride, over a period of 1 hr.

[1] It is not implied that the United States Department of Agriculture recommends the above company or its products to the exclusion of others in the same business.

The mixture was then dialyzed against water and lyophilized to give 2.1 g of material.

*Free amino groups.* Free amino groups of acylated β-casein A preparations were determined by reaction with ninhydrin, according to Fraenkel-Conrat (2). The color developed was compared directly to that of β-casein, to give the percentage of amino groups acylated. This percentage was also checked against the content of amino groups calculated from a standard curve procured by reaction of ninhydrin with leucine.

*DEAE-cellulose chromatography.* The method of Thompson and Kiddy (11) and Ribadeau-Dumas (9), using imidazole-urea buffer at pH 7.0, was employed. A DEAE-cellulose column 2 by 20 cm was prepared and NaCl gradients within the range of 0.0 to 0.35 M were utilized. The column effluent was monitored with a Canalco UV Analyzer and the absorbance at 278 mμ of appropriate tubes was then determined with a Beckman DU Spectrophotometer.[1]

*Zone electrophoresis.* Polyacrylamide gel electrophoresis in urea-Tris-EDTA-borate buffer, pH 9.1-9.3 (8, 12) was used to characterize the fractions obtained by DEAE-cellulose column chromatography and to evaluate the purity of the β-casein derivatives prior to chemical tests.

*Calcium sensitivity tests.* The method of Zittle and Walter (17) was used. Turbid supernatants were clarified by cooling. The absorbance at 278 mμ of the cooled supernatants and blanks was determined with a Beckman DU spectrophotometer.

*Sedimentation behavior.* One per cent solutions of protein in pH 6.86 potassium phosphate-KCl buffer, ionic strength 0.20 (14), were centrifuged at 52,640 rpm at 20.0 C in a Spinco Model E analytical ultracentrifuge.[1] These runs were performed by Mr. Eugene Barber.

## Results and Discussion

An electrophoretically homogeneous fraction of acetylated β-casein was obtained by DEAE-cellulose chromatography. The elution profile, shown in Figure 1, shows that the protein derivative began elution at a NaCl concentration near 0.13 M. Under the same conditions a slightly lower concentration of NaCl, 0.11 M, is required to start elution of β-casein (13). The material associated with the peak gave, after isolation, a single band following polyacrylamide gel electrophoresis (Figure 2). Mobility of the acetylated protein was greater than that for β-casein (Table 1). The extent of reaction of this preparation of acetylated β-casein with ninhydrin indicated that 82% of the amino groups of β-casein had been acetylated.

The elution profile for DEAE-cellulose chromatography of succinylated β-casein is shown in Figure 3. The NaCl concentration needed to begin elution was 0.20 M, appreciably higher than that required by β-casein under the same conditions (0.11 M). From the chromatographic fraction in Figure 3 was obtained a single, fast-moving band after PAG electrophoresis (Figure 2, Table 1). The extent of reaction of ninhydrin with the succinylated β-casein corresponding to the main peak in Figure 3 indicated that 86% of the amino groups had been succinylated.

The intensity of the stained bands in the polyacrylamide gel (Figure 2) fell off in the order of β-casein, acetylated β-casein, and succinylated β-casein. This series reflects the influence of net negative charge on acidic dye-binding capacity. As positive dye-binding sites

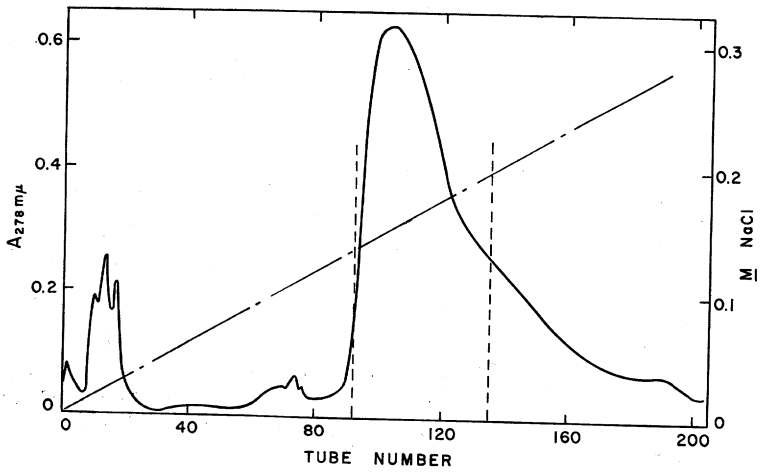


FIG. 1. DEAE-cellulose column chromatography of acetylated β-casein with 3.3 M urea, 0.01 M imidazole-HCl buffer, pH 7.0, at room temperature. To a 2- by 20-cm column was applied 0.7 g of material in 25 ml of buffer. A NaCl gradient from 0 to 0.3 M in two liters of buffer was used. The flow rate was 60 ml per hour (10 ml/tube).

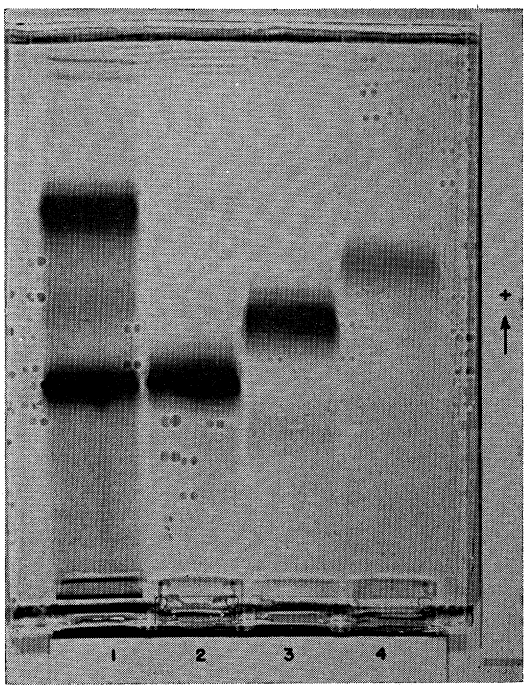


FIG. 2. Polyacrylamide gel electrophoresis in 4.5 M urea, pH 9.1 Tris-EDTA-borate buffer of DEAE-cellulose chromatographic fractions of β-casein and acylated β-caseins. Thirty microliters of 2% whole casein (1), of 1% β-casein (2), of 1% acetylated β-casein (3), and of 1% succinylated β-casein (4).

are removed through acetylation and, further, as dye-repulsing carboxylate groups are added through succinylation, the β-casein derivatives bind a smaller proportion of Amido Black.

The acetylated β-casein preparation exhibited greater stability to calcium ions near neutrality than did β-casein, as shown by results of calcium ion sensitivity tests in Table 2. The still greater stability of succinylated β-casein to calcium ions is established by the observation that a 0.3% solution of succinylated β-casein in 0.1 M calcium chloride at neutrality remained free of turbidity upon warming to 37 C.

Addition of a small proportion of κ-casein to a solution of acetylated β-casein increased the solubility of the latter in the presence of calcium ions (Table 2). This indicates that, since κ-casein stabilized acetylated β-casein against precipitation with calcium ions, all the amino groups of β-casein are not required for interaction of κ-casein with β-casein.

When β-casein was precipitated by calcium ions in the presence of succinylated β-casein, little or none of the derivative was co-precipitated, as judged by polyacrylamide gel electrophoresis of the precipitate. Therefore, interaction of molecules of β-casein with succinylated β-casein appears to be weak in comparison to interactions solely between molecules of β-casein.

Because both acetylated β-casein and succinylated β-casein failed to dissolve at pH values below the pH of precipitation, the stability of these derivatives to divalent anions in acidic solutions (15) could not be determined.

The sedimentation of β-casein gave two peaks at 1% concentration, pH 6.86, and ionic strength 0.20, with respective sedimentation coefficients of 1.32 S and 9.31 S (Figure 4). These values agree well with respective $s_{20}$ values of 1.36 S and 9.17 S obtained by Sullivan et al. (10) at an ionic strength of 0.25 under the same conditions otherwise. The $s_{20}$ value of 4.67 S for the fast-sedimenting peak from the acetylated β-casein solution may indicate that the aggregate formed is of lower weight than the aggregate formed by β-casein ($s_{20}$ = 9.31 S), providing no large conformation difference is involved. The relatively low $s_{20}$ value of 1.12 S for succinylated β-casein may reflect a hydrodynamic effect resulting from the increased negative electrostatic charge introduced through succinylation. For instance, the succinylated β-casein may be constrained to an extended conformation, to attenuate an increased negative charge. However, the main feature of the sedimentation of succinylated β-casein is that no fast-moving peak was obtained, which suggests that this derivative does not aggregate under conditions favoring aggregation of β-casein.

One explanation for the different $Ca^{++}$ sensitivity and sedimentation behavior of succinylated β-casein is that the added carboxyl groups, when ionized, prevent extensive aggregation of the protein by offering a large electrostatic repulsive force to neighboring molecules. In β-casein, aggregation is permitted by a balance of anionic and cationic groups of the protein. When this balance is upset through a lowering

TABLE 1

Electrophoretic behavior of acylated β-caseins

| | Relative mobility [a] | Extent of acylation [b] of $NH_2$-groups |
|---|---|---|
| | | (%) |
| β-Casein A | 0.65 [c] | .... |
| Acetylated β-casein A | 0.85 | 82 |
| Succinylated β-casein A | 1.05 | 86 |

[a] Polyacrylamide gel electrophoresis with urea-Tris-EDTA-borate buffer, pH 9.1-9.3 (8, 12).

[b] Determined by reaction with ninhydrin according to Fraenkel-Conrat (2).

[c] Value assigned by Thompson and Pepper (13).

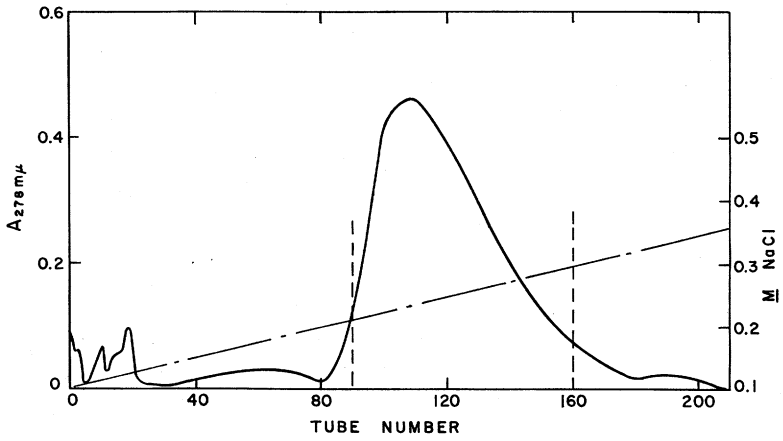


FIG. 3. DEAE-cellulose column chromatography of succinylated β-casein with 3.3 M urea, 0.01 M imidazole-HCl buffer, pH 7.0, at room temperature. To a 2- by 20-cm column was applied 0.7 g of material in 25 ml of buffer. A NaCl gradient from 0 to 0.35 M in two liters of buffer was used. The flow rate was 100 ml per hour (10 ml/tube).

of the pH to the isoelectric point or through the binding of calcium ions near neutrality, then aggregation proceeds to an extent which causes precipitation. Acetylation of β-casein removes cationic amino groups and raises the concentration of $Ca^{++}$ needed for precipitation of the protein-$Ca^{++}$ complex. Succinylation of β-casein, in addition to removing cationic groups, also adds anionic carboxylate groups which introduce negative charges. The concomitant reduction of aggregation, therefore, presumably results from an increase in hydrophilic ionic sites and from an increase in intermolecular electrostatic repulsive forces. Moreover, Gehrke et al. (3) have shown that β-casein does not aggregate at pH 8.6 at 20 C in Tris-citrate buffer (sedimentation coefficient of 1.6 S). Raising the pH from 6.86 to 8.6 increases the net negative charge of β-casein, because of deprotonization of imidazole groups. Thus, an electrostatic repulsion between molecules of β-casein at pH 8.6 may prevent aggregation, just as an electrostatic repulsion between molecules of succinylated β-casein at pH 6.86 may prevent aggregation.

That the formation of intermolecular salt bridges by carboxylate groups and calcium ions is responsible for precipitation of β-casein by calcium ions at 30 C near neutrality is not supported by the marked decreased sensitivity to calcium ions of succinylated β-casein. This modified β-casein, having 10 to 11 extra carboxylate groups per molecule, would be able to form a larger proportion of salt bridges with calcium ions and, therefore, on this basis would be expected to be more readily precipitated by calcium ions. Apparently then, intermolecular salt bridges between carboxylate groups and calcium ions are not primarily responsible for precipitation of β-casein by calcium ions. This conclusion is in accord with the finding by Waugh and co-workers (5, 6), that calcium ions are bound largely to phosphate groups of $\alpha_{s1,2}$-casein. However, since Pepper and Thompson (7) have shown that dephosphorylated $\alpha_s$-casein can still be precipitated by calcium ions at pH 7.0, sites other than phosphate groups are available for binding of calcium ions. In β-casein, calcium ions are probably bound to the same types of sites, i.e., phosphate groups and possibly carboxylate groups.

The removal by acylation of cationic protonated amino groups of β-casein prevents solu-

TABLE 2

Calcium ion sensitivity of acylated β-caseins

| | Concentration protein (%) | Concentration $CaCl_2$ (mM) | Soluble portion at 30 C (%) | Final pH [a] |
|---|---|---|---|---|
| β-Casein | 0.15 | 25 | 0 | 6.6 |
| Acetylated β-casein | 0.15 | 25 | 44 | 6.6 |
| Acetylated β-casein + κ-casein | 0.15<br>0.01 | 25 | 94 | 6.7 |
| Succinylated β-casein | 0.20 | 33 | 100 | 6.3 |

[a] pH obtained after mixture of neutral solutions (pH 7.0-7.5) and centrifugation.

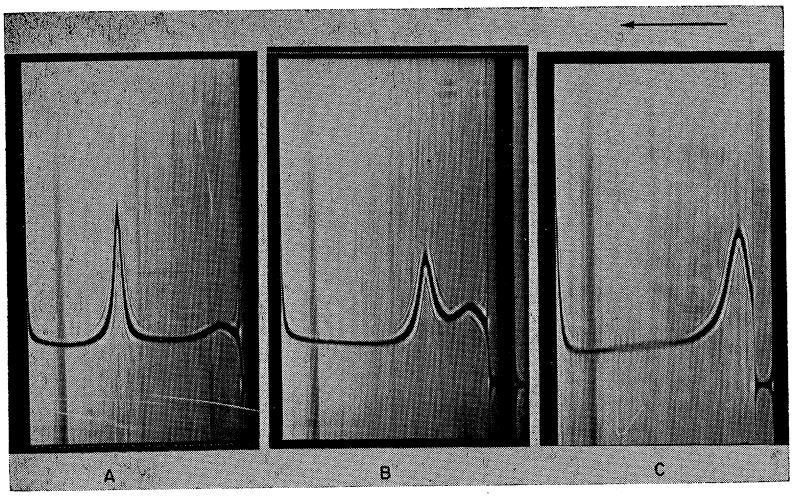


FIG. 4. Sedimentation patterns at pH 6.86, 20 C, and ionic strength of 0.20, after 64 min at 52,640 rpm. (A) β-Casein, 1.32 S and 9.31 S, (B) acetylated β-casein, 1.48 S and 4.67 S, and (C) succinylated β-casein, 1.12 S.

tion of the modified β-casein below the isoelectric point. Presumably, an insufficient number of hydrophilic cationic groups are present to overcome aggregation, which probably results largely from hydrophobic bonds involving the alkyl and aromatic groups of constituent amino acid residues.

The alteration of physical properties of β-casein produced by acylation of amino groups reflects the contribution of electrostatic forces to the physical state of β-casein in solution. Moreover, electrostatic interactions and hydrophobic bonding probably have complementary roles in the structure of the casein micelle in milk.

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